1　　**Perfluorinated Alkyl Acids and Fecundity Assessment in Striped mullet**
2　　**(*Mugil cephalus*) at Merritt Island National Wildlife Refuge**
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Abstract

This study investigated wild caught Striped mullet (*Mugil cephalus*) at Merritt Island National Wildlife Refuge (MINWR) for levels of 15 perfluoroalkyl acids (PFAAs) in tandem with fecundity measurements (n = 42) and oocyte developmental stages (n = 128). PFAAs measurements were undertaken for liver (n = 128), muscle (n = 49), and gonad (n = 10). No significant negative impacts of liver PFAA burden on wild-caught, mullet fecundity endpoints are observed in this study; however, changes in PFAAs are seen in the liver as mullet progress through different sub-stages of oocyte development. Of the PFAAs with significant changes by sub-stage of oocyte development, the carboxylic acids (perfluorooctanoic acid, perfluorononanoic acid, and perfluorotridecanoic acid) increase in the liver with increasing sub-stage while the sulfonic acid and its precursor (perfluorooctanesulfonic acid (PFOS) and perfluorooctanesulfonamide, respectively) decrease in the liver with increasing stage of oocyte development. This is a unique find and suggests PFAAs change location of compartmentalization as mullet progress towards spawning. Investigations also revealed higher than expected median muscle and gonad levels of PFOS in Striped mullet collected at MINWR (9.01 ng/g and 80.2 ng/g, respectively).

Keywords: PFOS, teleost, fecundity, PFOA, wildlife

Highlights

• High liver PFOS in Striped mullet (median, 124 ng/g; range, 12.6 – 2770 ng/g)

• Liver PFOA, PFNA, & PFTriA increase with increasing oocyte development

• Liver PFOS and PFOSA decrease with increasing oocyte development

• No significant negative impacts of liver PFAA on wild-caught, mullet fecundity

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7   1. Introduction

8   Perfluoroalkyl acids (PFAAs) is a commonly studied family within the larger group of

9   chemicals known as perfluoroalkyl substances (PFAS). PFASs are organic chains (branched and

10   linear) in which all hydrogen atoms attached to the carbon backbone have been substituted for a

11   fluorine atom creating a carbon fluoride (C-F) bond. Two subclasses of the PFAA family that will

12   be investigated in this study are perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkyl

13   sulfonic acids (PFSAs). Structurally, PFCAs and PFSAs have the general chemistry formula

14   $C_nF_{2n+1}COOH$ and $C_nF_{2n+1}SO_3H$, respectively (Buck et al., 2011).

15   With numerous applications in waterproofing, stain proofing, and firefighting products

16   (Moody and Field, 2000; Kärrman et al., 2011; de Solla et al., 2012; Place and Field, 2012; Laitinen

17   et al., 2014), PFAAs have found their way into the environment (de Solla et al., 2012), humans

18   (Laitinen et al., 2014), and wildlife (Houde et al., 2011) across the globe. Recent investigations of

19   PFAA levels in the American alligator (*Alligator mississippiensis*) in Florida and South Carolina

20   revealed variations in PFAA burden by site, noting that alligators residing at Merritt Island

21   National Wildlife Refuge (MINWR) maintained the highest PFAA burden compared to alligators

22   present at other southeastern sampling sites (Bangma et al., 2017a). This would suggest that

wildlife around MINWR are at higher risk to potential exposure to PFAAs in comparison to other investigated sites within Florida and South Carolina.

PFAAs have shown a variety of health effects such as immunotoxicity (DeWitt et al., 2012), neurotoxicity (Liao et al., 2009), and reduced fertility and fecundity. These reduced fecundity rates, due to PFAAs exposure, have been observed in human (*Homo sapiens*) (Fei et al., 2009; Velez et al., 2015), copepod (*Tigriopus japonicas*) (Han et al., 2015), nematode (*Caenorhabditis elegans*) (Tominaga et al., 2004), and freshwater flea (*Hyalella azteca*) (Lee et al., 1986) studies, while some human (Whitworth et al., 2012) and zebra fish (*Danio rerio*) (Wang et al., 2011) studies have shown no adverse effects of the investigated PFAAs on fecundity.

The pathways for possible mechanisms of action are still being elucidated for many of these effects. Some are PPAR dependent (Ren et al., 2009) and some are PPAR independent (Ren et al., 2009; Rosen et al., 2010). While PPARα is expressed in grey mullet (*Chelon labrosus*) liver and gonad tissue (Raingeard et al., 2006), potential PPAR independent mechanisms for changes in fecundity and fertility in teleosts have begun to be investigated as well. Changes in liver histology has been recorded in both male and female zebra fish exposed to perfluorooctanesulfonic acid (PFOS) (Cui et al., 2017), as well as changes in expression of vitellogenic genes recorded in tilapia hepatocytes (Liu et al., 2007) and zebra fish (*Brachydanio rerio*) livers (Cheng et al., 2012). In the case of the tilapia hepatocytes, the changes in expression of vitellogenic genes depended upon co-exposures with estrogen. While most of these studies have been conducted in a controlled laboratory setting, it is possible PFOS and other PFAAs may impact or change a female teleost's fecundity through impacts on the liver and gonad in the wild.

To date, no study published has attempted to measure potential fecundity effects in a wildlife population. MINWR is ideally suited to investigate potential wildlife fecundity effects of PFAAs due to the higher levels of PFAAs measured in wildlife (American alligators) compared to other locations in Florida and South Carolina (Bangma et al., 2017a). Therefore, this study aimed to investigate PFAA levels and fecundity measures in a locally abundant marine species that is prey species of alligators at MINWR and is also consumed by local fishermen in the surrounding areas outside of MINWR. Of the several fish species present at MINWR that met both of these criteria, the Striped mullet (*Mugil cephalus*), was one of the fastest species to maturity and was also one of the few species that undergoes isochronal spawning. These qualities ensure minimal effect of sampling on the population and highly accurate fecundity measurements. Overall, this study aimed to investigate PFAA burden and fecundity endpoints in sexually mature, female striped mullet early in the spawning season at MINWR.

2. Materials and methods

2.1. Sample collection

Collections of striped mullet were conducted at MINWR under the protocol GRD-06-044 review by the Institutional Animal Care and Use Committee (IACUC). Sampling was conducted October 24-28 (n = 83) and December 4-7, 2016 (n = 45) to ensure that samples were collected during the time period where reproductive development was occurring for the spawning season (McDonough, 2003). Striped mullet were obtained from numerous locations throughout the Banana River (BR), as well as from the drainage ditch that runs the length of the Shuttle Landing Facility (SLF) (Supplemental Information (SI), Figure S1). Unlike the fish in the Banana River, that were free to move about the entirety of the river system, the fish within in the SLF were trapped within the SLF drainage ditch and were unable to move outside of that area for years at a

1   time (only during infrequent large flood events can mullet move in and out of the SLF). Fish were

2   caught using a cast net (n = 125) as the primary form of sampling gear with a few adult mullet (n

3   = 3) obtained using a 183-m haul seine. Samples obtained using a 183-m haul seine are a result of

4   collaborations with Florida Marine Research Institute (FMRI). Of the mullet captured, only adult

5   female mullet larger than 30 cm were collected for this study to ensure that a high percentage of

6   sampled mullet had reached sexual maturity (McDonough, 2005). Sex was assessed in the field by

7   applying pressure to the abdomen and looking for the extrusion of milt or eggs (Kucherka et al.,

8   2006).

9   All mullet identified as female were necropsied within 12 h of capture. Standard

10   morphological measurements taken were total length (TL), standard length (SL), fork length (FL),

11   total height (TH), and fish girth (FG) in cm, and fish weight (FW), liver weight (LW), and gonad

12   weight (GW) in grams (g) (SI, Figure S2). Fish girth was taken as fish circumference at the same

13   location fish height was measured. Any subsequent mention of fish length in the remaining text

14   will be total length unless otherwise noted. Sagittal otoliths were removed for estimating fish age.

15   Livers were collected in methanol rinsed foil and frozen a − 20 ℃ for later PFAA analysis. Gonads

16   were collected and divided for analysis. One large section from the distal end of the left gonad was

17   wrapped in methanol rinsed foil and frozen a − 20 ℃ for later PFAA analysis. The whole right lobe

18   of the gonad was weighed separately and preserved in 10% NBF for fecundity counts.

19   Additionally, a small section (~ 1cm^3) from the posterior portion of the gonad, where the lobes

20   were joined, was removed and fixed in 10 % neutral buffered formalin (NBF) for histological

21   confirmation of sex and reproductive stage.

2.2. Chemicals

Two solutions, National Institute of Standards and Technology (NIST) Reference Materials (RMs) 8446 Perfluorinated Carboxylic Acids and Perfluorooctane Sulfonamide in Methanol and RM 8447 Perfluorinated Sulfonic Acids in Methanol were combined to create calibration solutions for liquid chromatography-tandem mass spectrometry (LC-MS/MS). analysis. The final solution comprised of 15 PFAAs as follows: perfluorobutyric acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTriA), perfluorotetradecanoic acid (PFTA), perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), PFOS, and perfluorooctanesulfonamide (PFOSA).

All internal standards (IS) employed in this study were purchased from Cambridge Isotope Laboratories (Andover, MA), RTI International (Research Triangle Park, NC), and Wellington Laboratories (Guelph, Ontario), to create an internal standard (IS) mixture that was comprised of a total of eleven isotopically labeled PFAAs. The IS mixture is as follows: [13C4]PFBA, [13C2]PFHxA, [13C8]PFOA, [13C9]PFNA, [13C9]PFDA, [13C2]PFUnA, [13C2]PFDoA, [18O2]PFBS, [18O2]PFHxS, [13C4]PFOS, and [18O2]PFOSA.

NIST Standard Reference Material (SRM) 1946 Organic Contaminants in Lake Superior Fish Tissue were co-analyzed as control materials during PFAA and tissue chemistry analysis (www.nist.gov/srm/). The PFAA levels of SRM 1946 processed during our extraction met established values reported on the Certificate of Analysis. Measured compounds were considered

above the reporting limit (RL) if the mass of an analyte in the sample was greater than the mean

plus three standard deviations of all blanks.

2.3. Sample preparation

Briefly, approximately 1 g of tissue samples (n_{liver} = 128, n_{muscle} = 49, n_{gonad} = 10),

calibrants, blanks, and SRM 1946 were extracted twice using 2.5 mL 0.01 mol/L KOH in methanol

after being spiked with approximately 600 µL of the IS mixture (Reiner et al., 2011a). All samples,

blanks, SRMs, and calibrants were further purified in methanol using an Envi-carb cartridge

(Supelco, Bellefonte, PA) and analyzed by LC-MS/MS.

Samples were analyzed using an Agilent 1100 High Performance Liquid Chromatography

system (HPLC; Santa Clara, CA) coupled to an Applied Biosystems API 4000 triple quadrupole

mass spectrometer (Applied Biosystems, Foster City, CA) with electrospray ionization in negative

mode. An Agilent Zorbax Eclipse Plus C18 analytical column (2.1 mm x 150 mm x 5µm) was

used for separation of PFAAs. Each individual sample run involved a ramping LC solvent gradient

with methanol and de-ionized water both containing 20 mmol/L ammonium acetate (Reiner et al.,

2011b). To ensure no interferences, two multiple reaction monitoring (MRM) transitions for each

PFAA were employed. For all PFAAs measured, one MRM was employed for quantitation and

the other transition was used for confirmation of the PFAA (Reiner et al., 2011b).

2.4. Histological processing and staging

Gonad tissues were processed using standard histological techniques (Humason, 1967) and

embedded in paraffin and sectioned. Sections were placed on microscope slides and stained with

standard haematoxylin and eosin-Y staining techniques. Histological criteria used to determine

reproductive stage has been previously established by McDonough et al. (McDonough, 2005)

(Table S1). Mullet captured in this study fell into three stages: Stage 2: Developing (n = 84), Stage 4: Atretic or spent (n = 2), and Stage 5: Inactive or resting (n = 42). Stage 2 encompasses a wide range of developing oocytes sizes and vitellogenic stages and therefore was separated into sub-stages for analysis: 2-early, 2-mid, and, 2-late (**Table 1, Figure S3**)

2.5. Fecundity

Fecundity determinations were made for 42 mullet in the 2-late sub-stage of oocyte development (**Table 1**). The 400 µm threshold has previously been established as the benchmark at which oocytes to be spawned were identifiable (Shehadeh et al., 1973). Striped mullet are isochronal spawners, so all developing oocytes would be spawned in a single event (McDonough, 2003). Fecundity was estimated using a modified gravimetric method as published by McDonough et al. (McDonough, 2003).

The fixed right gonad lobe was patted dry and re-weighed. The ovarian lobe was sampled three times creating three sub-samples for each mullet in the study: one at the posterior, one in the middle, and one at the anterior portion of the gonad. Three sub-samples were taken to account for any differential oocyte density throughout the ovarian lobe (McDonough et al., 2003). These sub-samples were preserved in 70 % isopropanol until oocyte counts could be conducted. Sub-sample weights ranged from 0.011 g to 0.031 g. The sub-samples from each specimen were then teased apart, spread along a Bogorov tray, and counted using a dissecting microscope at 10X magnification. Each sub-sample was counted twice and averaged. If counts varied greater than 10 %, a third count was performed. Oocyte density was calculated by dividing the mean number of oocytes by the mean weight of all three sub-samples for each mullet. The oocyte density was then used to calculate the total oocyte number for each ovary through expansion estimates using the whole gonad weight to produce a measure of fecundity.

2.6. Aging

Age was determined using the sagittal otoliths. After being embedded in epoxy resin, a 0.5-mm traverse section was cut with a low speed saw with diamond wafering blades. The thin section of the otolith was viewed at 20x magnification. The number of annular rings present were recorded as a proxy for age.

2.7. Statistics

Statistical analysis were preformed using IBM SPSS Statistics 22 (Armonk, NY: IBM Corp.). Parametric tests were used when data was normally or log-normally distributed and non-parametric tests were employed when data was non-normal. Statistical tests were performed for any PFAA that was detected in 75 % or more of samples measured for a tissue type (muscle, liver, gonad). The remaining PFAA were excluded from statistical analysis. For those PFAAs included in statistical analysis, compounds less than the RL were set equal to half the RL prior to running the statistical tests (Keller et al., 2005). Generalized linear regression models were created for investigations into the relationship between PFAA and fecundity as well as stage and PFAAs. Significant co-variates like fish weight and total length were included in the models while non-significant co-variants like age were excluded.

3. Results and discussion

3.1. Basic morphometrics

Female mullet collected at MINWR during October and December varied in total length from 30.9 cm to 51.8 cm with a normal distribution and a mean of 42.1 cm total length. Mullet collected in December (n = 45) were significantly longer than mullet collected in October (n = 83) (p < 0.001) (SI, Table S2, Figure S4). Sub-stages of collected mullet varied greatly with mullet collected in October showing a wider range of sub-stages than mullet collected in December (SI<

Figure S5). All mullet in sub-stage 2 late (n = 42) used for fecundity measurements were collected

in December 2016. The age of collected mullet ranged from 1 to 6 years of age and did not differ

significantly by month of collection (Mann Whitney U, p =0.126) (SI, Figure S6). As has been

seen by McDonough and colleagues, total length and fish weight were highly correlated for female

Striped mullet (p < 0.001, r = 0.959, n = 128) (SI, Figure S7) (McDonough, 2003, 2005).

3.2. PFAA detection

Nine the 15 PFAAs investigated were detected regularly (> 75 % of the time) in the mullet

livers (n = 128), and they are as follows (in order of abundance): PFOS, PFHxS, PFUnA, PFDA,

PFNA, PFDoA, PFOA, PFOSA, and PFTriA (**Table 2**). PFTA was detected in 46 % of the

samples, and the remaining PFAAs investigated were below RL in all liver samples. PFOS was

regularly detected in mullet muscle (n = 49), while PFDA, PFNA, PFHxS and PFUnA were

detected infrequently in muscle samples (67 %, 61 %, 13 %, and 5 %, respectively). All remaining

investigated PFAAs were below RL in muscle samples. Five PFAAs were regularly detected in

mullet gonad ($n_{gonad} = 10$), and were as follows (in order of abundance): PFOS, PFHxS, PFDA,

PFUnA, and PFNA. In addition, PFDoA was detected in 10 % of the gonad samples and the

remaining PFAAs were below the RL in gonad.

3.3. PFAA correlations

Correlations between the various measured PFAAs within the mullet liver were

investigated (**Table 3**) to determine if similar PFAA trends were observed in mullet liver compared

to alligator plasma at MINWR. All significant correlations between the various measured PFAAs

were found to be positive. The highest correlations within the liver were between PFUnA and

PFDA (r = 0.883). Similar to correlations between PFAAs in MINWR alligators (Bangma et al.,

2017b), some of the higher correlations in mullet liver were generally found between PFUnA,

PFDA, PFDoA, and PFOS. Other PFAAs, like PFOA and PFNA, are highly correlated in mullet liver as well, similarly to the alligators at MINWR. This would suggest these correlation similarities could be due to exposure from similar sources at or around MINWR or from alligators consuming the mullet MINWR and taking on a similar burden profile.

In addition, PFAA correlations between tissues were briefly investigated in this study. PFOS was the only PFAA measured over RL in 75 % or more of the muscle samples, so PFOS correlations between liver and muscle were examined. Liver to muscle PFOS correlation was highly significant ($p < 0.0001$, $r = 0.959$, $n = 49$). Therefore, a measure of PFOS in muscle tissue can predict PFOS in the liver tissue with high accuracy (95.9 %) and vice versa. On average liver PFOS was 12 times higher than muscle PFOS.

Even though PFAAs were measured in only 10 gonads from the mullet sampled for this study, correlations between liver and gonad PFAA were briefly investigated. Of the five PFAAs measured regularly over RL in the gonads (**Table 2**), significant correlations were found for PFNA ($p = 0.011$, $r = 0.757$, $n = 10$) and PFDA ($p = 0.019$, $r = 0.721$, $n = 10$). PFAA measurements in the remaining mullet gonads ($n = 118$) should be investigated in the future to improve upon the strength of correlations determined here, as well as, possibly revealing additional correlations that were missed due to small sample numbers ($n = 10$).

3.4. PFAAs by location

Since mullet were captured from two distinct locations, the SLF and BR, we investigated differences in PFAA burden by location of capture. First, levels in mullet liver by location (SLF liver: $n = 20$, BR liver: $n = 108$) were investigated. Significant differences between SLF and the BR were found for PFOS ($p < 0.001$), PFHxS ($p < 0.001$), and PFDoA ($p = 0.022$) (Figure 1) with

mullet in the SLF maintaining higher liver burdens than mullet in the BR. On average PFOS, PFHxS, and PFDoA are 4, 3.5 and 2 times higher in the SLF livers than the BR livers, respectively. This is not an unexpected result because the SLF site has held fire training events nearby using AFFFs in the past. In addition, AFFFs are a mixture of PFAAs, most notably PFOS and PFHxS, and alligators captured in the SLF region has shown high levels of plasma PFOS (Bangma et al., 2017b).

PFAAs by location were also investigated in the muscle tissue of the collected Striped mullet. Since higher levels of certain PFAAs were observed in livers from SLF compared to BR, all 20 SLF collected mullet were included in the muscle analysis, and 29 randomly selected mullet were included from the BR. Muscle tends to maintain lower levels of PFAAs than the liver in most vertebrates, and that was the case for the MINWR mullet. Only PFOS, PFDA, and PFNA were measureable above RL in 50 % or more of the muscle samples (n = 49) (**Table 2**), of those three, PFOS ($p < 0.001$) was the only PFAA to show a significant difference by location of capture (SI, Figure S8).

The levels of PFOS in mullet muscle exhibit a wide range (median, 9.48 ng/g; range, 1.93 ng/g − 95.3 ng/g). These values of PFOS in mullet muscle at MINWR are higher than expected since mullet are low on the aquatic food web, and certain PFAAs like PFOS are highest in top predator due to bioaccumulation up the food web (Houde et al., 2006; Muller et al., 2011). The only other species investigated for PFOS at MINWR was the American alligator (Bangma et al., 2017a; Bangma et al., 2017b). However, the studies investigating American alligator PFOS burden

1   at MINWR only investigated plasma, therefore are not directly comparable to the mullet muscle

2   values obtained in this study.

3   Levels of PFOS in Striped mullet muscle from MINWR were compared to Fish

4   Consumption Screening Values (FCSV) developed for the Michigan Fish Consumption Advisory

5   Program for PFOS in September of 2016 (Figure 2,

1   Table *4*) (Michigan Department of Health and Human Services, 2016). Michigan's FCSV is one

2   of the only regulations on PFOS consumption in fish tissue in the United States and is the most

3   recent regulation released to the public. Michigan's FCSV values are intended to be guidelines

4   for the general public in Michigan to delineate how often PFOS burdened fish should be consumed.

5   While no commercial harvesting occurs on MINWR grounds, mullet travel long distances and are

6   free to leave the BR into surrounding area where commercial fishing does occur, so this study will

7   compare MINWR muscle PFOS levels to Michigan's FCSV values.

8   MINWR mullet muscle PFOS levels fall into a variety of the Michigan's FCSV categories

9   ranging from 16 meals per month to once a month (Figure 2,

Table *4*). Mullet collected from the SLF consistently fell into stricter consumption

categories compared to BR mullet. This follows logically with the significantly higher PFOS in

SLF mullet muscle compared to BR mullet muscle. For the most part, mullet inside the SLF cannot

make it to the BR except in the event of extreme flooding events which occur infrequently. No

mullet collected from either the SLF or BR at MINWR fell into the "Do Not Eat" category. One

interesting note, there is no 'no limit' category at the low end of PFOS muscle burden. The FCSV

states this is due to

> "the still emerging information on health effects from PFOS exposure, and
> background exposure to the general population, and potential health effects from
> exposure to multiple [perfluorinated substances]."

Like mullet muscle, female mullet gonads, also known as mullet roe, are consumed by

humans. Therefore, human exposure to PFAAs through roe consumption should be investigated,

especially since mullet gonad contains higher levels of PFOS than mullet muscle (**Table 2**). No

consumption advisories have been created for mullet roe due to the lack of knowledge on

prevalence and portion size of mullet roe consumption. Since a roe consumption advisory does not

exist, this study compared roe PFOS levels for the 10 gonads measured in this study to the

Michigan FCSV for fish muscle. The comparison revealed that even among the 10 mullet gonad

measured from BR in this study, one fell into the category six meals per year (Figure). This would

indicate that even mullet collected from the BR, have levels of PFAA in their roe that is a concern

when it comes to consumption. No significant correlation between PFOS in the gonad and PFOS

in the liver were found, therefore, in order to estimate the levels of PFOS in the remaining collected

mullet roe, further chemical extraction and analysis of mullet gonad from BR and SLF is required.

3.5. PFAAs and fecundity

Of the 128 sampled female striped mullet, 42 collected in December had eggs in sub-stage 2 late that were included in the fecundity assessment A generalized linear model for fecundity was created for each liver PFAA investigated (detected in over RL in >75 % of samples). Each model included total length and fish weight which were significant covariates and excluded all other non-significant covariates such as age. While both fish weight and total length were significant, fish weight was more highly significant compared to total length in the model. No significant interaction was found between liver PFAAs and total length, and liver PFAAs and fish weight with one exception: PFHxS and fish weight ($p = 0.0373$).

Out of the nine liver PFAAs, PFNA, PFDA, and PFUnA were significantly related to fecundity with increasing liver PFAA leading to increasing number of eggs (**Table 5**). In addition, PFUnA, PFDoA, PFTriA, and PFOSA all trended ($0.05 < p < 0.10$) to a similar pattern as PFNA, PFDA, and PFUnA with increasing PFAAs and increasing total eggs. These results highlight that carboxylic acids ranging from 9 to 11 carbons show a stronger relationship than the longer carboxylic acids with greater than 12 carbons. While no sulfonic acid showed significance, PFOSA, a precursor to PFOS, trended towards significance.

We hypothesize that increasing PFAA is related to increasing number of eggs in this study because mullet with higher total length and greater fish weight would consume more food than smaller mullet. The consumption of more food would lead to both an increase in energy for production of more total eggs (fecundity) and an increase in the consumption (and accumulation) of PFAAs via diet. So an increase in PFAAs with increasing eggs is not directly related where one is causative to another, but rather, both are affected by the mullet's diet. Unlike some laboratory studies, no significant negative impacts of PFAA on wild-caught, mullet fecundity endpoints are

observed in this study. While that is the case in this study, future aqua culture studies that control for diet fluctuations and dose at various levels of PFAAs may still reveal subtle links between PFAAs and fecundity in teleosts.

3.5. Sub-stage and PFAAs

All 128 female mullet collected for this study were staged for oocyte development and a model created to assess the relationship between sub-stage and liver PFAAs. Again, a generalized linear regression model was created and included significant covariates total length and fish weight while excluding all other non-significant covariates such as age. Sub-stages were investigated in this model due to the wide variety of developing oocytes sizes and vitellogenic stages found in stage 2. The progression of the histological changes within stage 2 are important to distinguish between because they correspond to physiological changes that might impact or be related to changes in PFAA levels. For this model, sub-stages were defined as an ordered variable. Since the mullet collected in this study were all of reproductively active age (no stage 1), stage 5 (resting) was considered a resting state prior to the 2016 spawning season, followed by stage 2 developing stages (early, mid, and late), stage 3, and finally progressing to stage 4 (atresia). Stages 3 and 4 were excluded from statistical analysis due to sampling sizes of 0 and 2, respectively.

Significant differences between sub-stages of oocyte development and liver PFAAs were discovered for PFOA, PFNA, PFTriA, PFOSA, and PFOS (**Table 6**). PFDoA also trended towards significance (p = 0.0655). The Parameter Estimate varies depending on the PFAA and seems to change depending on whether the PFAA is a carboxylic or sulfonic acid. Of the PFAAs with significant changes by sub-stage, the carboxylic acids (PFOA, PFNA, and PFTRiA) increase in the liver with increasing sub-stage of oocyte development while the sulfonic acid and its precursor (PFOS and PFOSA, respectively) decrease in the liver with increasing stage of oocyte

development. The liver is key in vitellogenesis for oocyte development in teleost, and these differences in PFAAs by sub-stage may reflect physiological changes in protein abundance in the liver and/or locations in various organs that show affinities for carboxylic acids and sulfonic acids.

Interaction terms were assessed for this model, and a number of significant interactions were found between sub-stage and fish weight, as well as for sub-stage and total length (SI, Table S3) for several PFAAs investigated (PFOA, PFNA, PFTriA, and PFOSA). A significant interaction value in this model indicates that larger fish (longer total weight and/or larger fish weight) are more advanced in sub-stages of oocyte development than smaller fish at the time of capture.

We hypothesize these interaction terms are likely due to the time of year the mullet were sampled. For example, all mullet from this study were sampled in late October and early December. This would place sampling for this study during the early portion of the mullet spawning season which runs from October through April (McDonough, 2003). During the spawning season, larger mullet tend to have more energy reserves and, therefore, develop oocytes earlier than smaller mullet. Therefore, this study collected of a variety of sized female mullet where the larger mullet were ahead in sub-stages of oocyte development compared to the smaller mullet due to the time of sampling. It is possible the interaction terms seen in this model would no longer be significant if mullet sampling events were taken at multiple time points that spanned the entire spawning season and not just the early spawning season.

4. Conclusions

This study revealed higher than expected muscle and gonad levels of PFOS in Striped mullet collected at MINWR. While no PFOS levels measured in tissue fell within the Michigan

FCSV "Do not eat" category for the consumption of fish muscle containing PFOS, many of the muscle and gonad (known as mullet roe) samples did fall within restriction levels ranging from between "16 meals a month" to only "1 meals a month." Fish from the higher restriction categories came from the SLF sampling area and are highly unlikely to reach commercial fisheries due to entrapment in the SLF compound.

This study also reveals changes in PFAAs in the liver (a key organ in vitellogenesis) as mullet progress through different sub-stages of oocyte development. Of the PFAAs with significant changes by sub-stage, the carboxylic acids (PFOA, PFNA, and PFTRiA) increase in the liver with increasing sub-stage of oocyte development while the sulfonic acid and its precursor (PFOS and PFOSA, respectively) decrease in the liver with increasing stage of oocyte development. This is a unique find and suggests PFAAs change location of compartmentalization as mullet progress towards spawning. This is likely due to changes in abundance and location of various proteins that have affinity for various PFAAs.

In addition, this study found an increase in PFAAs with increasing eggs (fecundity), however, increasing PFAAs is not directly related to increasing fecundity of the mullet. The mullets' diet represents a confounder variable in the study that cannot be removed without a more controlled experiment. Therefore, unlike some laboratory studies, no significant negative impacts of PFAA on wild-caught, mullet fecundity endpoints are observed in this study. Future aqua culture studies that control for diet fluctuations and dose at various levels of PFAAs may still reveal subtle links between PFAAs and fecundity in teleosts.

Table 1. Histological criteria used to determine reproductive sub-stage in stage 2 female striped mullet.

Reproductive sub-stage	
2 early	Developing oocytes are generally greater than 120 µm and smaller than 200 µm. Cortical alveoli are present but oocytes are still mostly pre-vitellogenic.
2 mid	Developing oocytes begin early stages of vitellogenesis ranging in size from 200 µm to 400 µm. Heterogeneous size structure of oocytes is common in this sub-stage. Nucleus is still visible.
2 late	Developing oocytes are all consistent in size and are in the late stages of vitellogenesis. At this sub-stage, oocytes are all at least 400 µm or larger in size, and nuclear migration to the pole has occurred.

Table 2. Perfluoroalkyl acid (PFAAs) concentrations (ng/g wet mass) in Striped mullet at MINWR.

Organ	PFAA	PFOSA	PFOS	PFHxS	PFOA	PFNA
Liver (n = 128)	% > RL	88	100	100	98	100
	Median	0.102	124	4.26	0.227	0.705
	Mean	0.285	192	6.81	0.329	0.996
	Max	2.05	2770	113	1.82	4.11
	Min	<0.009	12.6	0.386	<0.010	0.120
		PFDA	PFUnA	PFDoA	PFTriA	PFTA
	% > RL	100	95	93	100	46
	Median	2.07	1.98	0.385	0.217	<0.011
	Mean	2.31	2.16	0.542	0.263	<0.011
	Max	8.86	10.3	4.81	1.26	0.237
	Min	0.087	<0.008	<0.009	0.019	<0.008
		PFHxS	PFOS	PFNA	PFDA	PFUnA
Gonad (n = 10)	% > RL	90	100	100	100	90
	Median	1.25	80.2	0.476	0.642	0.941
	Mean	1.32	90.0	0.518	0.809	0.891
	Max	2.66	202	0.994	2.06	1.93
	Min	<0.035	33.5	0.166	0.303	<0.040
		PFOS	PFNA	PFDA		
Muscle (n = 49)	% > RL	100	61	67		
	Median	9.01	0.168	0.126		
	Mean	15.7	0.182	0.146		
	Max	95.3	0.315	0.504		
	Min	1.93	<0.124	<0.007		

Values were calculated with half the RL substituted for non-detects as described in the methods section, but values shown as "<" a specified number describe the actual RL.

Table 3. PFAA correlations in Striped mullet liver from MINWR (n = 128). All values are spearman's rank correlation coefficient rho for non-normal data except when indicated.

	PFNA	PFDA	PFUnA	PFDoA	PFTriA	PFOSA	PFHxS	PFOS
PFOA	.771**	.125	.007	-.129	.256**	-.331**	0.025	-0.065
PFNA		.407**	.206*	-.024	.452**	-.211*	.275**	***.333****
PFDA			.883**	.629**	.604**	.572**	.258**	.749**
PFUnA				.806**	.618**	.633**	.239**	.695**
PFDoA					.601**	.597**	0.133	.510**
PFTriA						.221*	-0.051	.296**
PFOSA							.213*	.560**
PFHxS								.615**

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Results from Pearson's for log normal data

Table 4. Fish Consumption Screening Values (FCSV) as defined by the State of Michigan in September of 2016 (Michigan Department of Health and Human Services, 2016) and number and percent of striped mullet that fall within each consumption class for all measured mullet muscle, SLF muscle, and BR muscle.

Muscle PFOS (ng/g)	Meals per month	Mullet (n = 49)		SLF (n = 20)		BR (n = 29)	
		n	%	n	%	n	%
≤ 9	16	24	49	3	15	21	72
> 9 to 13	12	8	16	3	15	5	17
> 13 to 19	8	4	8	1	5	3	10
> 19 to 38	4	9	18	6	30	3	10
> 38 to 75	2	3	6	3	15	0	0
> 75 to 150	1	1	2	1	5	0	0
> 150 to 300	6 meals per year	0	0	0	0	0	0
> 300	Do not eat	0	0	0	0	0	0

Table 5. Results of a generalized linear regression model for fecundity and PFAAs. p -values shown above represent a significant or non-significant change in total eggs (fecundity) with changing PFAA concentration (ng/g). Parameter Estimate represent the change in total eggs with 1 ng/g increase in PFAA concentration in mullet liver.

PFAA	p-value	Parameter Estimate
PFOA	0.136	109305
PFNA	0.025	87668
PFDA	0.033	53386
PFUnA	0.030	37765
PFDoA	0.067	61178
PFTriA	0.081	183035
PFOSA	0.066	290466
PFHxS	0.875	-2151
PFOS	0.317	534

Red indicates $p \leq 0.05$ while green indicates $0.05 \leq p \leq 0.10$

Table 6. Results of a generalized linear regression model for sub-stage and PFAAs. p-values
shown represent a significant or non-significant change in PFAAs with progressing egg
development (sub-stage). Parameter Estimate represent the change in PFAA with one increase
sub-stage development.

PFAA	Sub-stage & PFAA p-value	Parameter Estimate
PFOA	0.0064	0.08237
PFNA	0.0453	0.12196
PFDA	0.491	-0.11155
PFUnA	0.8563	0.02846
PFDoA	0.0655	0.11047
PFTriA	0.0496	0.03935
PFOSA	0.0009	-0.12658
PFOS	0.0387	-56.7088
PFHxS	0.1452	-1.59623

Red indicates p ≤ 0.05 while green indicates 0.05 ≤ p ≤ 0.10

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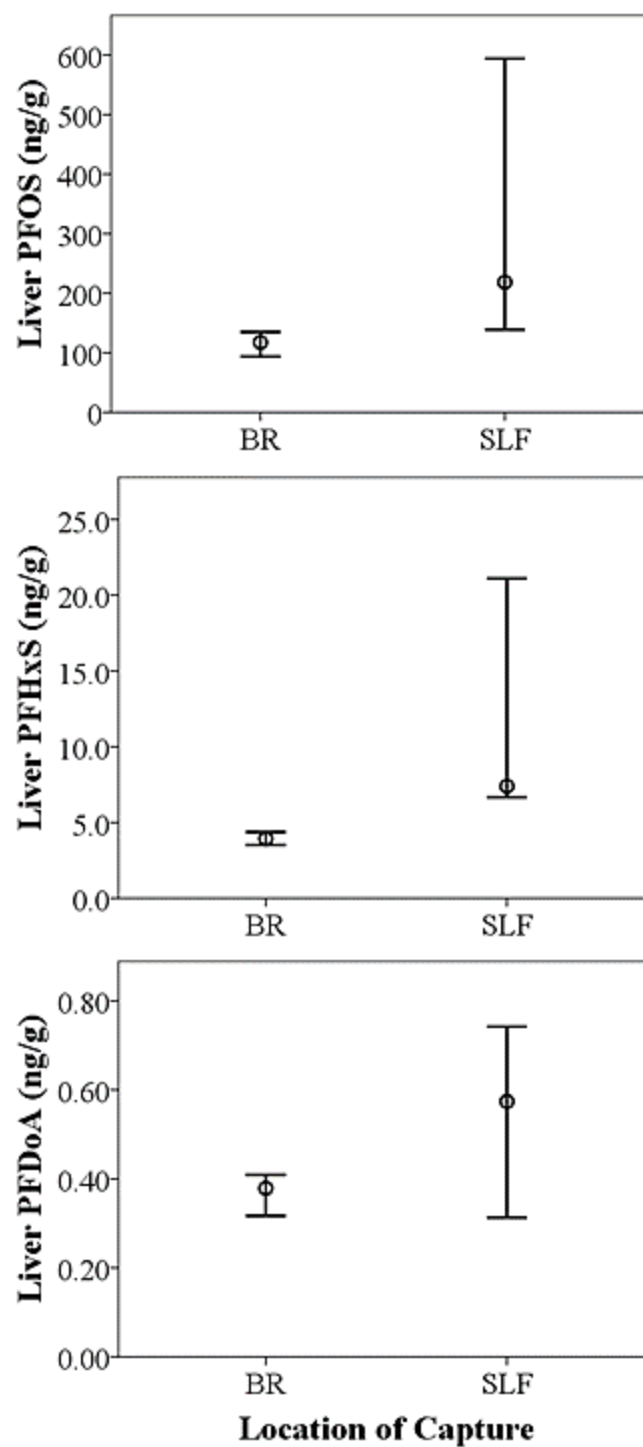
Figure 1. Median ng/g of PFOS (p <0.001), PFHxS (p <0.001), and PFDoA (p = 0.022) in mullet liver by location of capture (BR liver: n = 108, SLF liver: n = 20). Error bars represent 95% CI.

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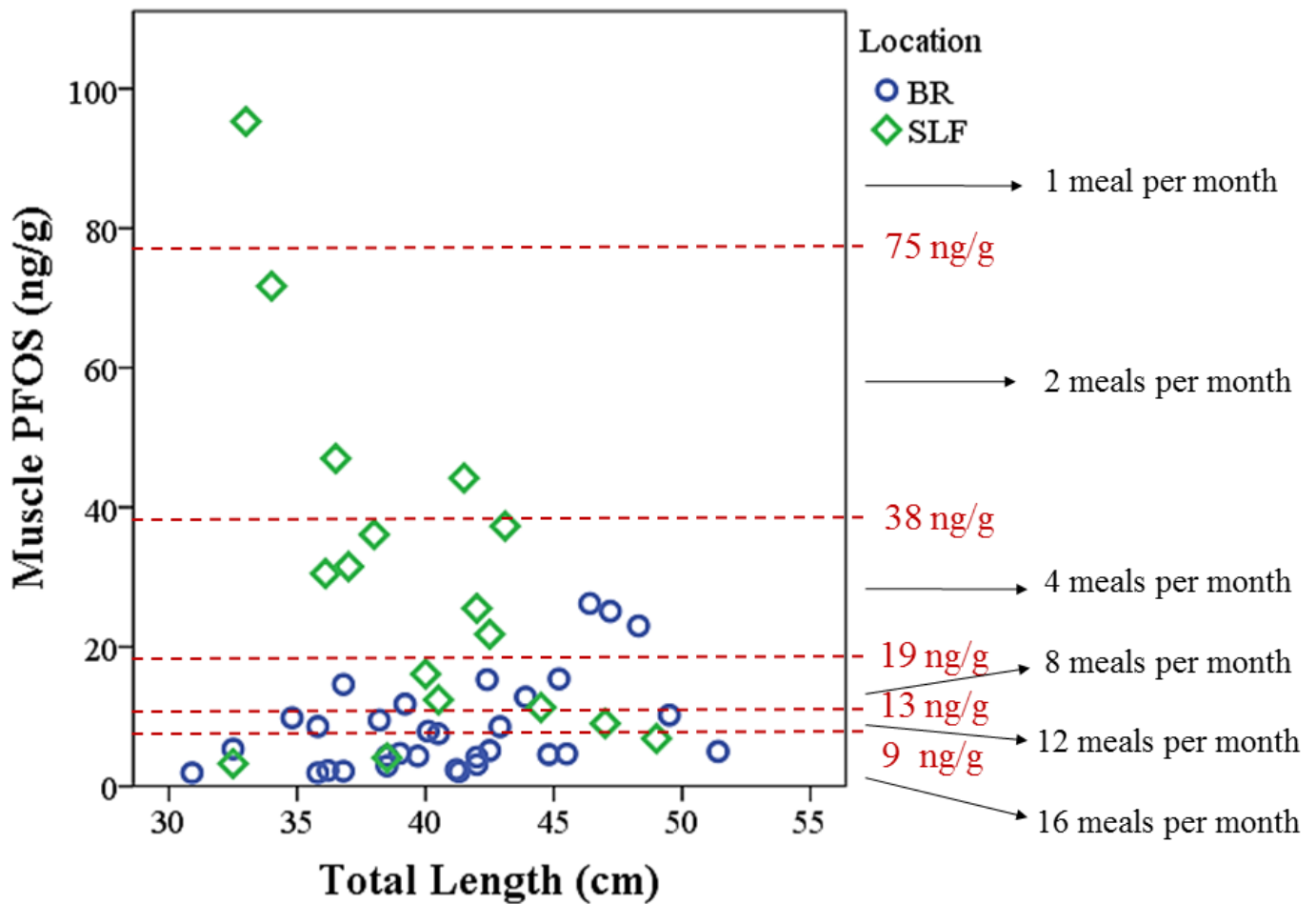
Figure 2. Individual mullet muscle PFOS levels compared to total length of fish. Fish
Consumption Screening Values (FCSV) developed for the Michigan Fish Consumption Advisory
Program (September 2016) are indicated (Michigan Department of Health and Human Services,
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Perfluorinated Alkyl Acids and Fecundity Assessment in Striped mullet (*Mugil cephalus*) at Merritt Island National Wildlife Refuge

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SUPPLEMENTAL INFORMATION

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Table S3. Interaction term p-values, parameter estimates, and standards for generalized linear regression model created to assess the relationship between sub-stage and liver PFAAs.

Figure S1. Collection sites for Striped mullet at Merritt Island National Wildlife Refuge.

Figure S2. Striped mullet pictorial representation of standard morphometric measurements for total length (TL), fork length (FL), standard length (SL), and fish height (FH).

Figure S3. Histological representations of sub-stages A) 2-early, B) 2-mid, C) 2-late used to determine reproductive sub-stage within stage 2 for all female striped mullet (n = 128).

Figure S4. Total length of Striped mullet collected at MINWR in October and December of 2016.
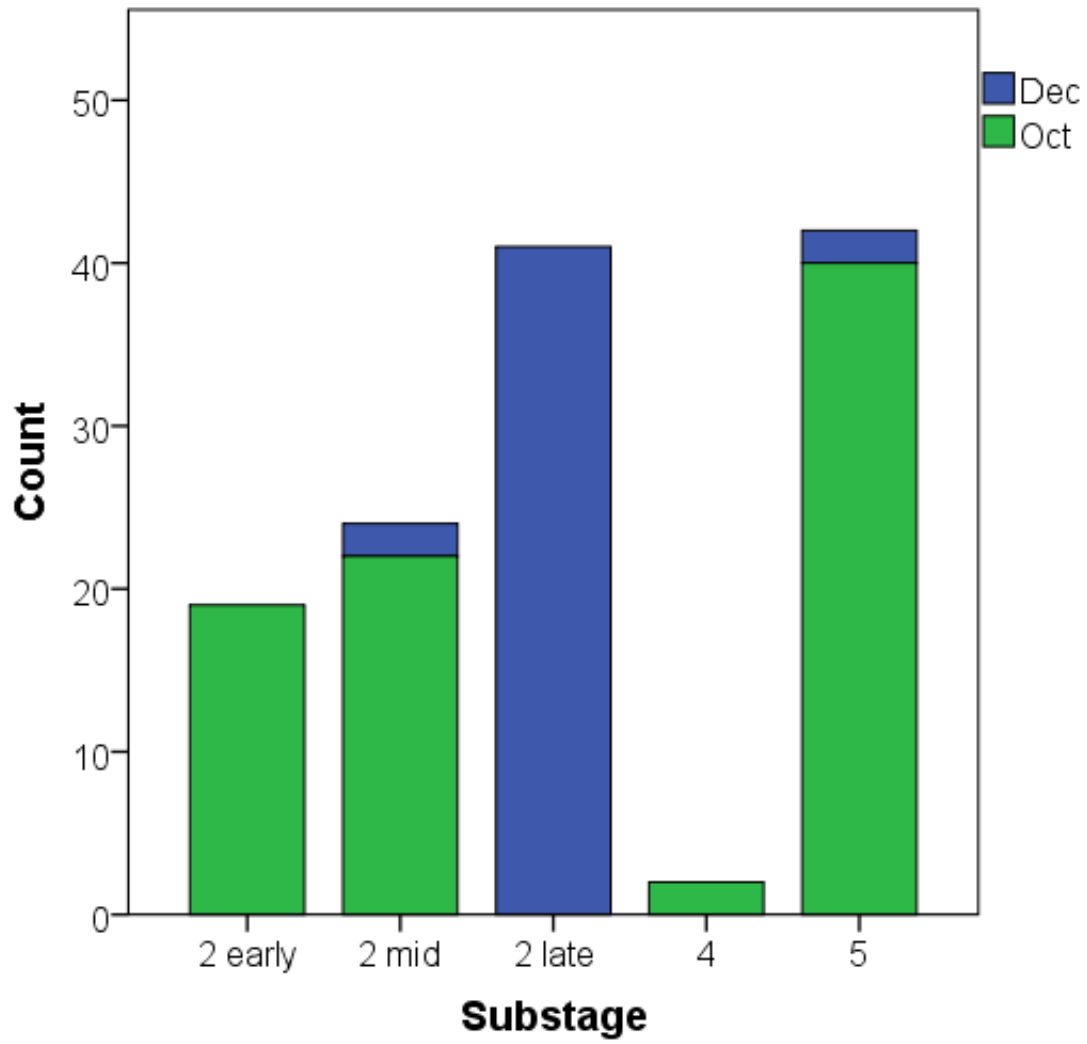
Figure S5. Sub-stages of Striped mullet collected at MINWR in October and December of 2016.
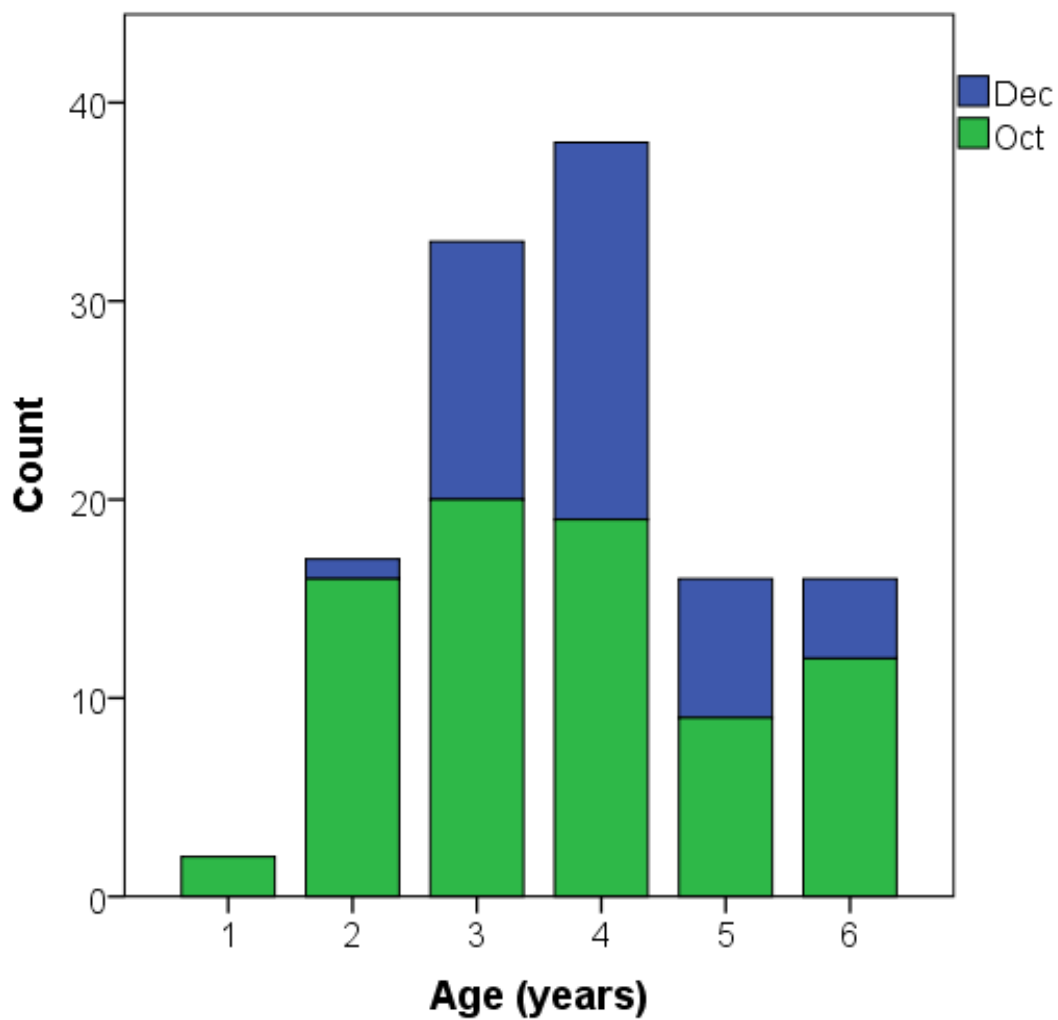
Figure S6. Age of Striped mullet collected at MINWR in October and December of 2016.

Figure S7. Fish weight and total length of Striped mullet collected at MINWR (Pearson's correlation: p < 0.001, r = 0.959, n = 128).

Figure S8. Median logPFOS (ng/g) in mullet muscle by capture location (p < 0.001). Error bars represent 95% CI.

Figure S8. Individual mullet gonad PFOS levels (location BR, n = 10) compared to total length of fish. Fish Consumption Screening Values (FCSV) developed for the Michigan Fish Consumption Advisory Program (September 2016) are indicated by red dashed lines (Michigan Department of Health and Human Services, 2016).

1   **Table S1.** Histological criteria used to determine reproductive stage in female Striped mullet.

Reproductive stage	
1. Immature	Inactive ovary with pre-vitellogenic oocytes and no evidence of atresia. Oocytes are <80 µm, lamellae lack muscle, and connective tissue bundles are not as elongate as those in mature ovaries, ovary wall is very thin.
2. Developing	Developing ovary have enlarged oocytes generally greater than 120 µm in size. Cortical alveoli become present and actual vitellogenesis occurs after oocytes reach 180 µm in size and continue to increase in size. Abundant yolk globules with oocytes reach a size range of >600 µm.
3. Running ripe	Completion of yolk coalescence and hydration in most oocytes.
4. Atretic or spent	More than 30% of developed oocytes undergoing the atretic process of breaking down and absorbing decaying cellular matter. Stains a distinct yellow-brown color.
5. Inactive or resting	Pre-vitellogenic oocytes with only traces of atresia. In comparison to those of immature females, most oocytes are > 80 µm, lamellae have some muscle and connective tissue bundles; lamellae are larger and more elongated than those of immature females and the ovarian wall is thicker.

2   (McDonough et al., 2005)

3

1 **Table S2.** Total length of female Striped mullet collected at MINWR in October and December of
2 2016.

	October	December	All Mullet
n	83	45	128
Median	40.0	45.8	42.0
Mean	40.4	45.2	42.1
Max	51.4	51.8	51.8
Min	30.9	37.4	30.9

3

Table S3. Interaction term p-values, parameter estimates, and standards for generalized linear regression model created to assess the relationship between sub-stage and liver PFAAs.

PFAA	Sub-stage & total length interaction p-value	Parameter Estimate	Sub-stage & fish weight interaction p-value	Parameter Estimate
PFOA	0.0335	0.0113	0.0193	-49.1738
PFNA	0.0001	0.0563	0.0001	0.001
PFDA	0.5184	0.0168	0.3024	0.0004
PFUnA	0.9416	0.0021	0.9492	0.0000
PFDoA	0.0812	-0.0199	0.0716	-0.0003
PFTriA	0.029	0.0078	0.033	0.0001
PFOSA	0.0002	-0.0238	0.0001	-0.0004
PFOS	0.2322	5.818	0.252	0.0933
PFHxS	0.1468	0.283	0.2028	0.0042

Red indicates $p \leq 0.05$ while green indicates $0.05 \leq p \leq 0.10$

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3   **Figure S1.** Collection sites for Striped mullet at Merritt Island National Wildlife Refuge.

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Figure S2. Striped mullet pictorial representation of standard morphometric measurements for total
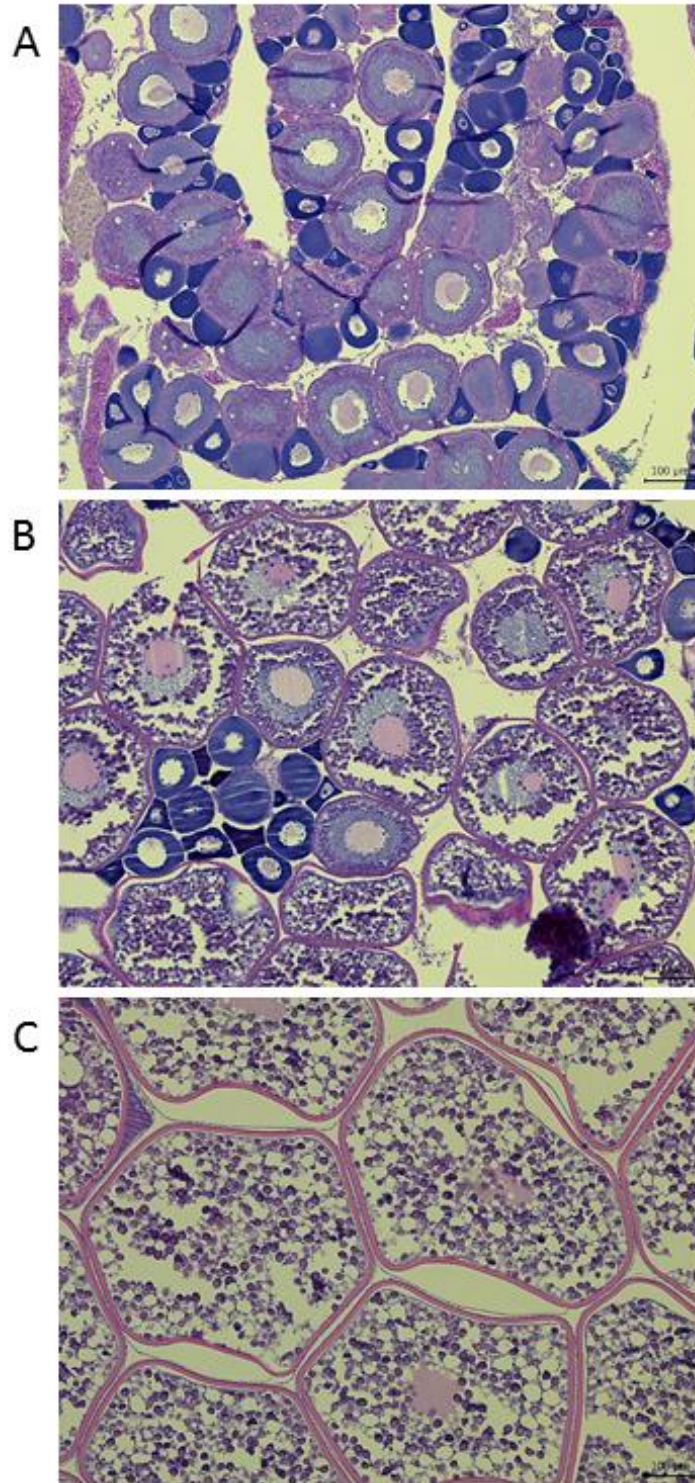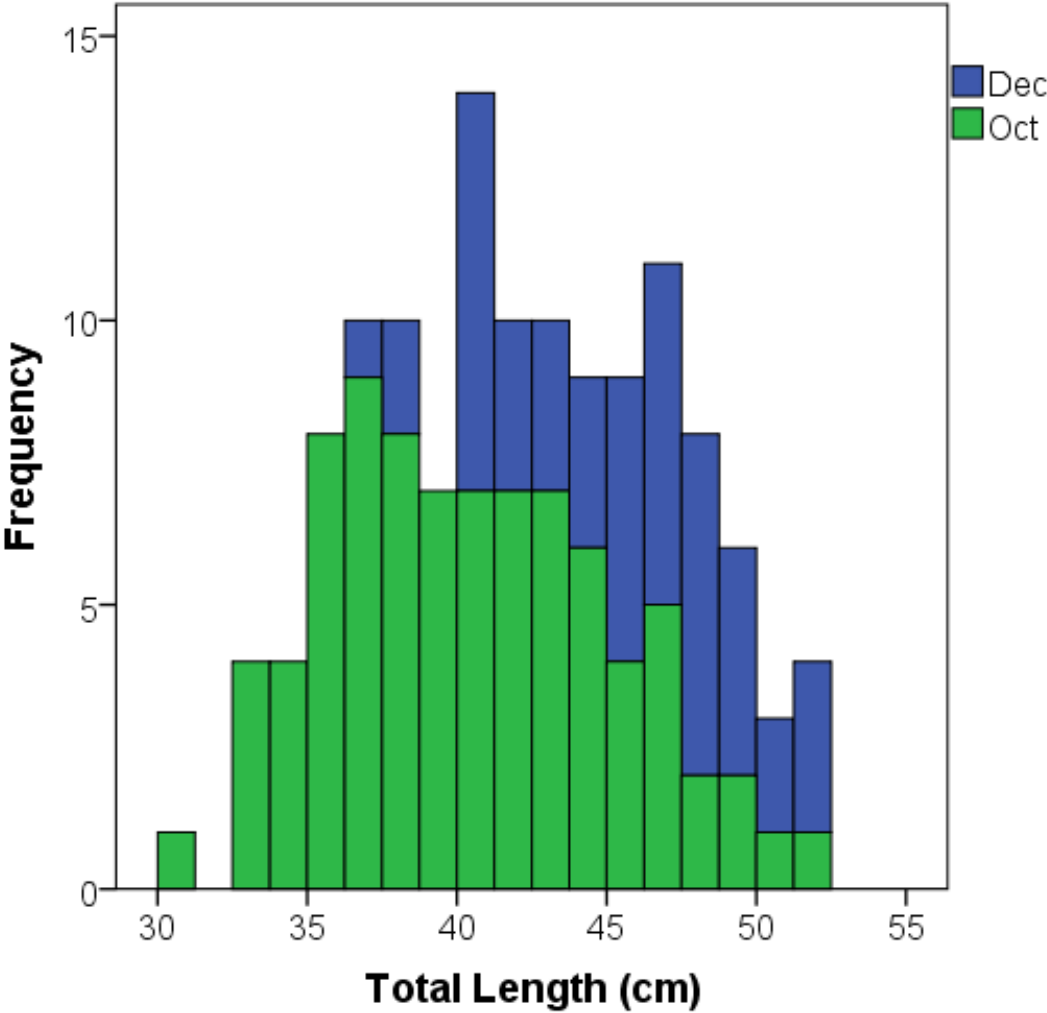length (TL), fork length (FL), standard length (SL), and fish height (FH).

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2 **Figure S3.** Histological representations of sub-stages A) 2-early, B) 2-mid, C) 2-late used to
3 determine reproductive sub-stage within stage 2 for all female striped mullet (n = 128).
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Figure S4. Total length of Striped mullet collected at MINWR in October and December of 2016.

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2 **Figure S5.** Sub-stages of Striped mullet collected at MINWR in October and December of 2016.
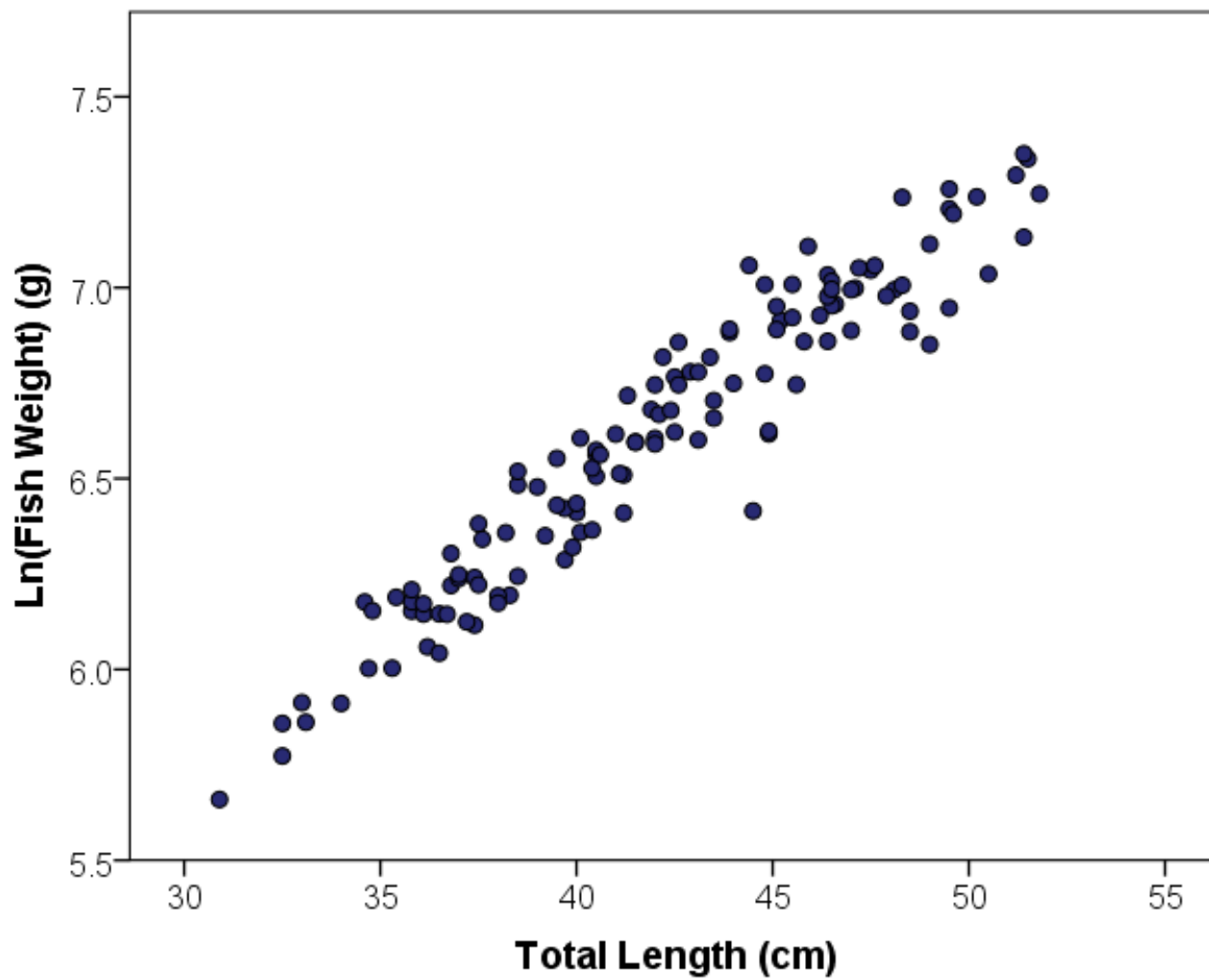
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3   **Figure S6.** Age of Striped mullet collected at MINWR in October and December of 2016.

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3 **Figure S7.** Fish weight and total length of Striped mullet collected at MINWR (Pearson's correlation:
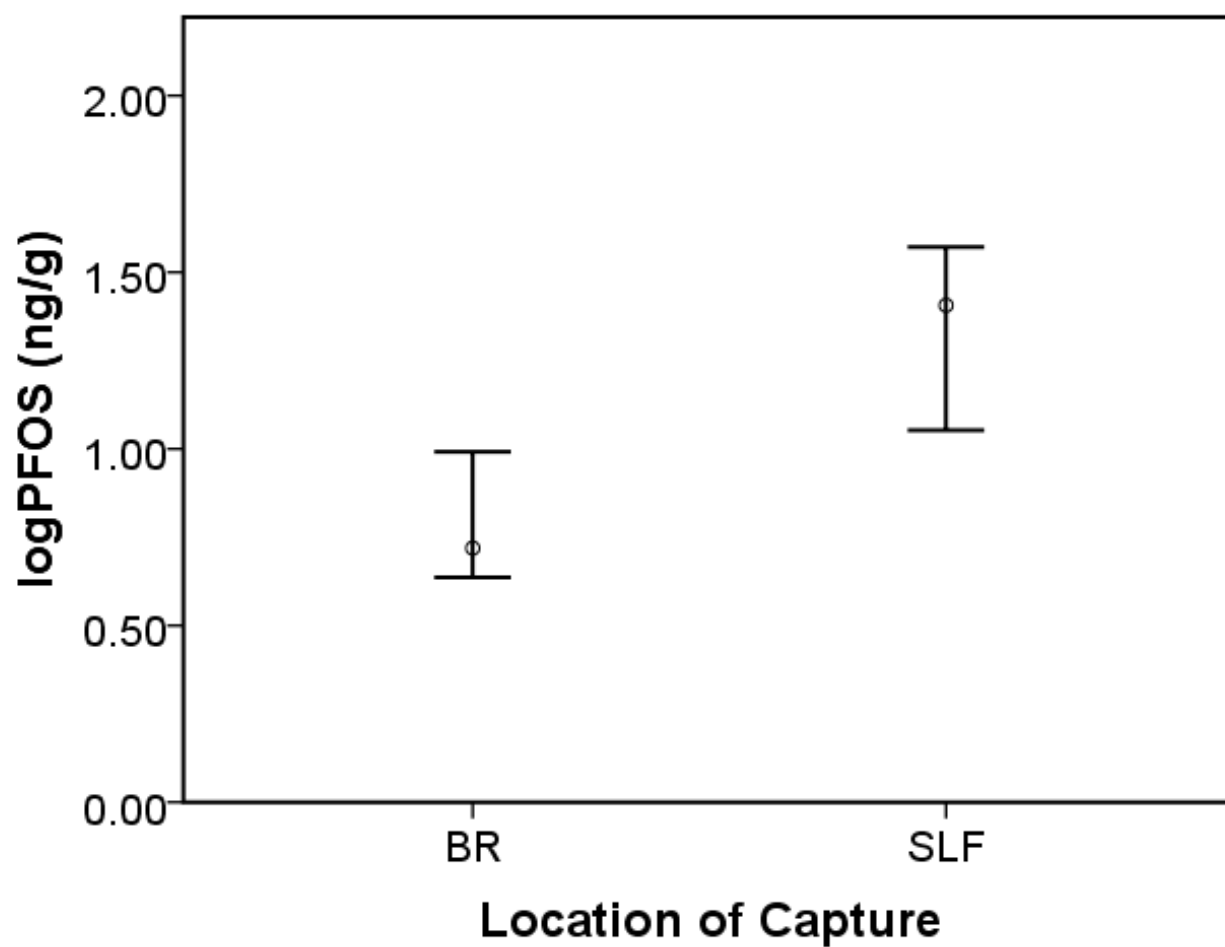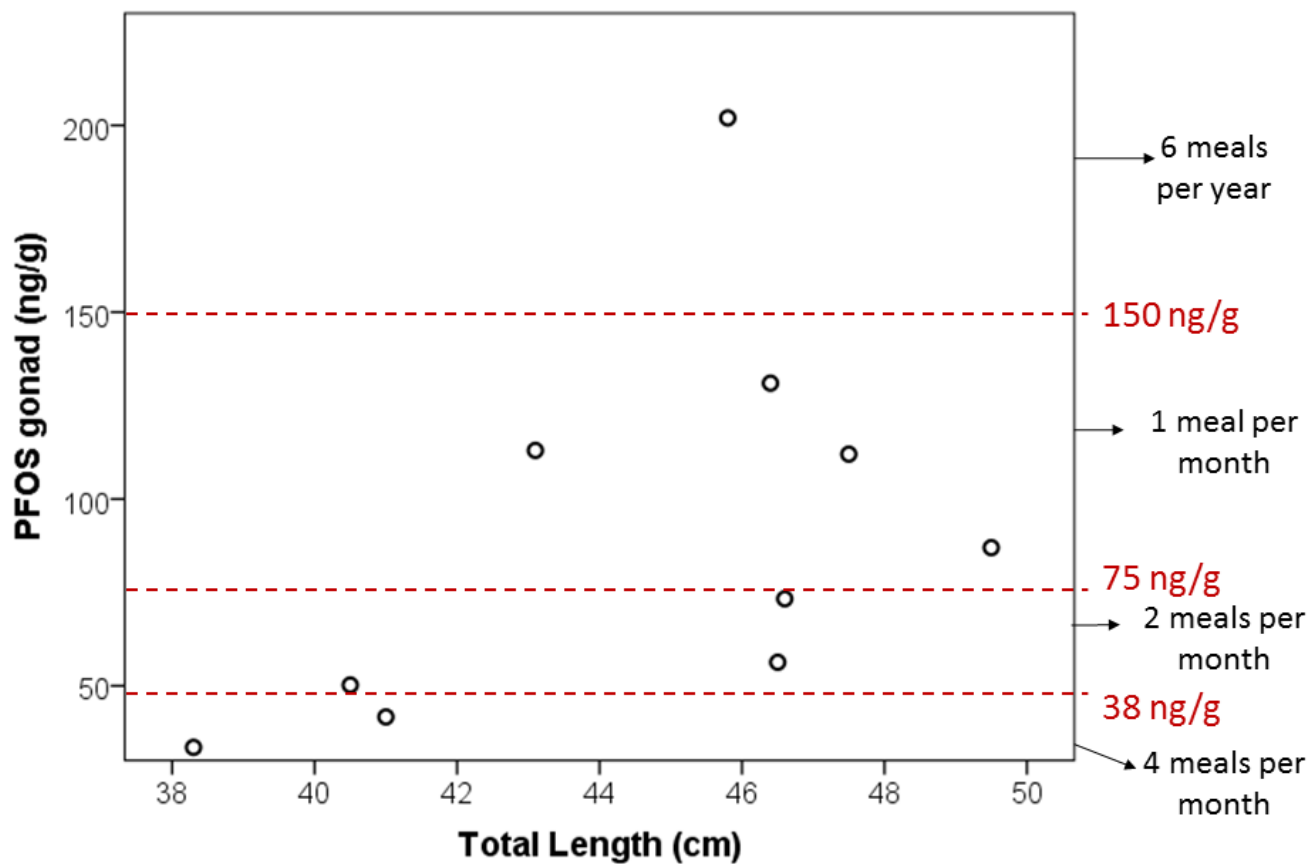4 $p < 0.001$, $r = 0.959$, $n = 128$).

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4 **Figure S8.** Median logPFOS (ng/g) in mullet muscle by capture location ($p < 0.001$). Error bars
5 represent 95% CI.
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Figure S8. Individual mullet gonad PFOS levels (location BR, n = 10) compared to total length of
fish. Fish Consumption Screening Values (FCSV) developed for the Michigan Fish Consumption
Advisory Program (September 2016) are indicated by red dashed lines (Michigan Department of
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